Non-genomic effects of progesterone on the signaling function of G protein-coupled receptors

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Abstract Progesterone at concentrations between 10 μM and 200 μM affected the calcium signaling evoked by ligand stimulation of G protein-coupled receptors expressed in several cell lines. At 160 μM progesterone the signaling of all receptors was completely abolished. The effect of progesterone was fast, reversible and was not prevented by cycloheximide indicating its non-genomic nature. Overall, the action of progesterone was more cell type-specific than receptor-specific. Our results are in contrast to a recent report [Grazzini, E., Guillon, G., Mouillac, B. and Zingg, H.H. (1998) Nature 392, 509–512] in which a direct high-affinity interaction between the oxytocin receptor and progesterone was suggested.

Key words: Progesterone; Non-genomic effect; G protein-coupled receptor; Calcium signal; Anisotropy

1. Introduction

Steroid hormones such as progesterone influence cell functions by binding to intracellular receptors which act as transcription factors in regulating protein synthesis. However, over the years, non-genomic effects of steroid hormones have become more and more evident (reviewed in [1]). Examples of progesterone-induced non-genomic effects are the inhibition of cholesterol biosynthesis [2], the initiation of meiosis in prophase-arrested amphibian oocytes [3] or the stimulation of the human sperm acrosome reaction [4]. In human sperm, for instance, progesterone produces a number of effects which resemble the action of extracellular ligands such as peptide hormones: a rapid increase of intracellular calcium concentration, tyrosine phosphorylation of proteins, efflux of chloride, stimulation of activity of phospholipases [4], activation of protein kinase C [5] and of extracellular signal-regulated kinases (ERKs) [6]. In order to initiate oocyte maturation or to inhibit cholesterol biosynthesis, progesterone concentrations in the micromolar range are necessary.

During pregnancy, progesterone maintains uterus quiescence, i.e. it reduces the sensitivity of this organ to oxytocin, a uterotonic neurohypophyseal hormone. However, the underlying mechanism of this effect is unclear. It has been proposed that non-genomic progesterone effects contribute to the maintenance of pregnancy [7,8]. In particular, a direct high-affinity interaction of progesterone and the oxytocin receptor has been postulated [8]. Grazzini et al. claimed that progesterone could act as a negative allosteric modulator of the oxytocin receptor and thus offered a plausible mechanism of how progesterone could contribute to uterine quiescence [8].

To address this in more detail, we investigated in several cell lines the influence of progesterone on the function of the oxytocin receptor and other G protein-coupled receptors (GPCRs). Hormones which activate the phosphoinositide cascade lead to a rapid increase of the cytosolic calcium concentration. We show that progesterone is able to markedly affect the calcium signaling function of GPCRs by a non-genomic mechanism. Overall, the action of progesterone was more cell type-specific than receptor-specific. Possible physiological implications are discussed.

2. Materials and methods

2.1. Materials

[*Tyrosyl*-2,6-^{3}H]oxytocin (NET-858, 48.5 Ci/mmol) was from NEN Du Pont de Nemours (Bad Homburg, Germany). RU-486 was a gift from Schering, Berlin, Germany. The ligands oxytocin, bradykinin, vasopressin, cholecystokinin-8, and the peptide SFLLRNP were from Bachem (Heidelberg, Germany). All other ligands, steroids, chemicals and culture media were purchased from Sigma (Deisenhofen, Germany).

2.2. Cell cultures and transfections

The following cell lines were used: human embryonic kidney cells (HEK293) untransfected (HEK293) or stably transfected with the human oxytocin receptor (HEK-OTR), the human V_1 vasopressin receptor (HEK-V_1R) or the human cholecystokinin$_B$ receptor (HEK-CCKR), 1321N1, a human astrocytoma cell line, and Chinese hamster ovary cells (CHO) untransfected (CHO-K1) or stably transfected with the human oxytocin receptor (CHO-OTR). The 1321N1 astrocytoma cell line was a generous gift from Dr. Toews (Nebraska, USA). HEK293 and CHO-K1 were stably transfected as described [9]. HEK293 and 1321N1 astrocytoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% and 5% fetal calf serum, respectively. CHO cells were cultured in DMEM/F-12 Ham supplemented with 10% fetal calf serum.

2.3. Membrane preparation and receptor binding assays

Membranes of HEK-OTR and CHO-OTR cells were prepared as described [9] and resuspended in binding buffer (20 mM HEPES, pH 7.4 and 5 mM $MgCl_2$). Membranes (150 μg) were incubated with 10 nM [^{3}H]oxytocin in a total volume of 100 μl binding buffer for 30 min at 30°C. The binding reaction was stopped by addition of ice-cold binding buffer and bound ligand was separated from free ligand by rapid filtration over Whatman GF/C filters. Non-specific binding was determined in the presence of a 500-fold excess of unlabeled oxytocin.

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Abbreviations: AVP, (Arg^8)-vasopressin; $[Ca^{2+}]_i$, intracellular free calcium ion concentration; CCK-8, cholecystokinin octapeptide (26–33) sulfated; CHO, Chinese hamster ovary; DPH, 1,6-diphenyl-1,3,5-hexatriene; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; PBS, phosphate-buffered saline

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2.4. Measurement of intracellular free calcium ion concentration $[Ca^{2+}]_i$

Cells were loaded with 1.5 μM fura-2/AM for 30 min at 37°C. They were detached using PBS/0.5 mM EDTA and centrifuged at $100\times g$ for 10 min. The pellet was washed and resuspended in calcium buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose) at a concentration of 2×10^6 cells/ml. Aliquots of the suspension (1×10^6 cells) were transferred into a thermostated (37°C) cuvette containing 2.5 ml calcium buffer. Ligands were applied to the cells and the changes of the $[Ca^{2+}]_i$ were monitored spectrofluorimetrically. The $[Ca^{2+}]_i$ was calculated by using the ratio 340/380 nm (emission at 510 nm).

2.5. Treatment of cells and membranes with steroids

All steroids were dissolved in ethanol. The steroids were added to fura-2/AM-loaded cells in calcium buffer (final concentration of ethanol $<0.8\%$ (v/v)). The cells were incubated for 10 min at 37°C and subsequently for 10 min at 25°C prior to their use for calcium measurements. In a further series of experiments, the steroids were added to membranes of HEK-OTR or CHO-OTR cells in binding buffer (final concentration of ethanol $<1\%$ (v/v)). Membranes were first incubated for 10 min at 30°C and were subsequently used for the ligand binding assays.

2.6. Measurement of steady-state anisotropy

Steroid-treated cells in calcium buffer (1×10^6 cells/ml) were sonicated on ice using a Branson sonifier. The cell lysate was labeled with 2 μM 1,6-diphenyl-1,3,5-hexatriene (DPH) for 60 min at 30°C to obtain maximum fluorescence intensity. The polarization measurements were performed as described [9].

3. Results

3.1. Determination of receptor subtypes

Ligand-induced increase of the intracellular free calcium ion concentration was measured in various cell lines using the following ligands at the indicated concentrations: acetylcholine (100 μM), bradykinin (1 μM), histamine (100 μM), oxytocin (100 nM), (Arg^8)-vasopressin (AVP) (100 nM), cholecystokinin (CCK-8) (100 nM), and the peptide SFLLRNP (1 μM). The peptide SFLLRNP is an agonist of the thrombin receptor which is endogenously expressed in CHO cell lines. In the following cell lines an increase of $[Ca^{2+}]_i$ was elicited by the indicated ligands: HEK293 (acetylcholine, bradykinin); HEK-V_1R (acetylcholine, bradykinin, AVP); HEK-CCKR (acetylcholine, bradykinin, CCK-8); HEK-OTR (acetylcholine, bradykinin, oxytocin); CHO-K1 (CCK-8, SFLLRNP); CHO-OTR (CCK-8, SFLLRNP, oxytocin); 1321N1 (acetylcholine, bradykinin, histamine). HEK293 and 1321N1 cells endogenously express the M_3 subtype of the muscarinic acetylcholine receptor and the B_2 subtype of the bradykinin receptor, 1321N1 cells express the H_1 subtype of the histamine receptor as indicated by their pharmacological profile (data not shown).

3.2. Effect of progesterone on calcium signaling of different GPCRs in various cell lines

Pretreatment of 1321N1 cells or different HEK293 cell lines with progesterone (10–200 μM) attenuated the agonist-stimulated signaling of all receptors investigated (Fig. 1A,B). At 160 μM progesterone, the signaling of all receptors was completely abolished. The IC_{50} values (μM progesterone) were as follows: (1) 1321N1 cells: acetylcholine receptor, 125 ± 2.5; bradykinin receptor, 103 ± 8.3; histamine receptor, 117 ± 3.2; (2) HEK293 cell lines: acetylcholine receptor, 73 ± 6.7; bradykinin receptor, 53 ± 11.3; oxytocin receptor, 56 ± 6.1; vasopressin receptor, 77 ± 6.2; cholecystokinin receptor, 86 ± 4.3.

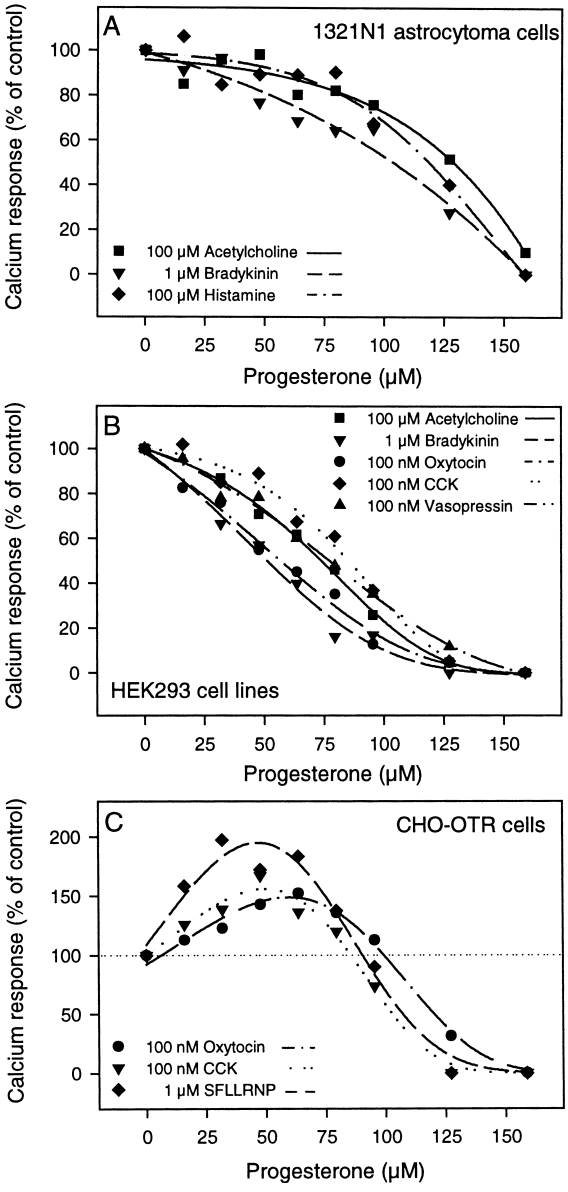

Fig. 1. Effects of increasing concentrations of progesterone on the ligand-induced calcium signaling of G protein-coupled receptors in (A) 1321N1 astrocytoma cells, (B) HEK293 cell lines and (C) CHO-OTR cells. Data are expressed as percent of control, i.e. 'ethanol-treated' cells. The basal $[Ca^{2+}]_i$ was between 50 and 100 nM (depending on the cell line). Following ligand application the $[Ca^{2+}]_i$ of control cells increased 2–5-fold over basal value (depending on both ligand and cell line).

Acetylcholine receptors in the different HEK293 cell lines revealed no significant differences with respect to the IC_{50} values of progesterone. This allowed us to directly compare various GPCRs in the different HEK293 cell lines. Overall, the different receptors revealed IC_{50} values of progesterone which were lower in HEK293 cells as compared with 1321N1 cells. This suggests a more cell type-specific action of progesterone. This assumption was confirmed in experiments with CHO-OTR cells. Surprisingly, the highest ligand-induced calcium signal was obtained when the cells were pretreated with about 50 μM progesterone (Fig. 1C). At progesterone concentrations higher than 60 μM, the calcium signals were dose-dependently diminished. As observed in HEK293 and 1321N1 cells, the

calcium responses were completely abolished when the cells were pretreated with 160 μM progesterone. Side effects of the transfection leading to this conspicuous behavior of CHO cells could be excluded, since progesterone had the same effect on the peptide SFLLRNP-induced calcium signaling in transfected (CHO-OTR) versus untransfected CHO-K1 cells (not shown).

3.3. Kinetics of the progesterone action

The calcium response of acetylcholine-stimulated 1321N1 cells was measured at different times after addition of 160 μM progesterone to the cells (Fig. 2A). After 3 min, the calcium response was reduced to 30% of control (i.e. ethanol-treated cells). Incubation of the cells with progesterone for more than 11 min prevented the calcium response completely. Next, we analyzed whether this progesterone effect was reversible. After removal of progesterone by centrifugation and a washing step with progesterone-free calcium buffer, the calcium signals increased to a level of 50% of control after 13 min. Finally, the 100% control level was regained after about 60 min (Fig. 2B). The effect of progesterone was not prevented when the cells had been pretreated with cycloheximide (160 μM) for at least 4 h at 37°C (not shown). Taken together, the action of progesterone is fast and reversible and not affected by an inhibitor of protein synthesis indicating a non-genomic pathway.

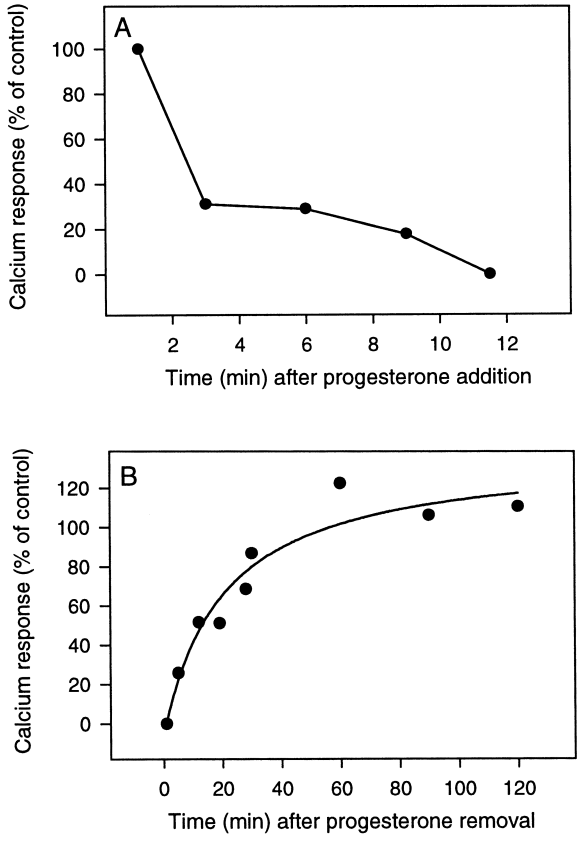

Fig. 2. Kinetics of progesterone action. The calcium response of 1321N1 astrocytoma cells on 100 μM acetylcholine was measured (A) at different times after addition of 160 μM progesterone to the cells and (B) after removal of progesterone. Data are expressed as percent of control, i.e. 'ethanol-treated' cells.

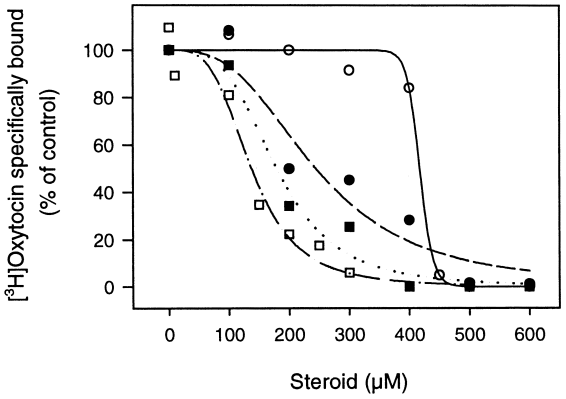

Fig. 3. Effects of progestins on the ligand binding activity of the human oxytocin receptor. Membranes of HEK-OTR/CHO-OTR cells were incubated with increasing concentrations of progesterone (○/□) or the progesterone metabolite 5β-pregnane-3,20-dione (●/■) for 10 min at 30°C. Subsequently, the membranes (150 μg each) were used for ligand binding assay as described. Data are expressed as percent of control, i.e. 'ethanol-treated' membranes (100% is equal to 4130 dpm and 2090 dpm of specifically bound [^{3}H]oxytocin for membranes of CHO-OTR and HEK-OTR cells, respectively). The data were fitted according to the equation: $y = 100/(1+10^{(\log(b)-\log(x))\times a})$, where b is the IC_{50} value and a is the slope factor. The slope factor values were as follows: −39.2 (○), −3.6 (□), −2.9 (●) and −3.6 (■). The IC_{50} values are shown in Section 3.4.

3.4. Effect of progesterone on the ligand receptor interaction

In further experiments we analyzed whether the observed effects of progesterone on the calcium signaling of GPCRs were due to an influence on the ligand–receptor interaction. As an example, we treated membranes of both CHO-OTR and HEK-OTR cells with increasing amounts of progesterone as well as the progesterone metabolite 5β-pregnane-3,20-dione and measured the specific binding of [^{3}H]oxytocin (Fig. 3). This progesterone derivative was used because Grazzini et al. previously reported an inhibition of [^{3}H]oxytocin binding to the human oxytocin receptor in membranes of CHO cells by 5β-pregnane-3,20-dione ($K_i = 32 \pm 5$ nM) but not by progesterone (up to 10 μM) [8]. In contrast to the results of Grazzini et al., we needed approximately 6000-fold higher concentrations of 5β-pregnane-3,20-dione to reduce the specific binding of [^{3}H]oxytocin. The required concentrations for half-maximal suppression were as follows: (1) progesterone: HEK-OTR membranes, 417 ± 4.2 μM; CHO-OTR membranes, 138 ± 6.9 μM; (2) 5β-pregnane-3,20-dione: HEK-OTR membranes, 244 ± 28.0 μM; CHO-OTR membranes, 180 ± 12.6 μM. The corresponding slope factors were found to be high for all curves (see legend to Fig. 3). This was most obvious for membranes of HEK-OTR cells treated with progesterone. This threshold-like effect may reflect alterations in the physical properties of the membrane bilayer such as fluidity changes which occur at such high progesterone concentrations (see Section 3.5).

3.5. Influence of progestins on the fluidity of membranes

Next, we investigated whether the progestin concentrations used in our experiments had an influence on the membrane fluidity. For this purpose, we measured the anisotropy of the fluorescent dye DPH in membranes of HEK-OTR cells treated with progesterone or 5β-pregnane-3,20-dione. At progestin concentrations of 60 μM, no changes of the anisotropy values were observed as compared with control, i.e. 'ethanol-

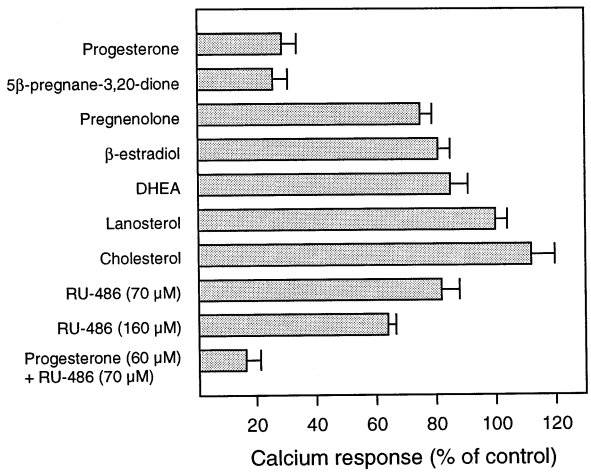

Fig. 4. Structure–activity analysis of steroids on receptor-mediated calcium signaling. HEK-OTR cells were incubated with different steroids (60 μM each, unless indicated otherwise) and the calcium response on 100 nM oxytocin was measured. Data are expressed as percent of control, i.e. 'ethanol-treated' cells.

treated' cells ($r = 0.1813 \pm 0.00327$). In contrast, when the membranes were treated with 450 μM progestin, a concentration at which an interference with the ligand–receptor interaction has been shown (see Section 3.4), the anisotropy values were slightly reduced (progesterone, $r = 0.1719 \pm 0.00064$; 5β-pregnane-3,20-dione, $r = 0.1752 \pm 0.00983$).

3.6. Structure–activity analysis of several steroids

To demonstrate the specificity of the progesterone action, various steroids were tested for their ability to diminish the ligand-induced calcium signaling. For this purpose, HEK-OTR cells were treated with different steroids (each at 60 μM). The steroids showed the following order of potency (oxytocin-induced calcium response in % of control, i.e. ethanol-treated cells, Fig. 4): progesterone ($29 \pm 5\%$) = 5β-pregnane-3,20-dione ($26 \pm 5\%$)≫pregnenolone ($75 \pm 4\%$)≥β-estradiol ($81 \pm 4\%$)≥dehydroepiandrosterone (DHEA) ($85 \pm 6\%$). Cholesterol slightly increased the calcium response on 100 nM oxytocin whereas its derivative lanosterol showed no effect. RU-486, a synthetic antagonist of the classical progesterone receptor, was less effective than progesterone. Treatment of cells with 70 μM or 160 μM RU-486 reduced the calcium response to $82 \pm 5.9\%$ and $64 \pm 2.6\%$ of control levels, respectively. In addition, RU-486 did not act as an antagonist of progesterone (Fig. 4).

4. Discussion

In this study, we demonstrate that progesterone can specifically affect the calcium signaling evoked by ligand stimulation of GPCRs. Several mechanisms that could account for the non-genomic effects of progesterone are not supported by our findings. (1) Progesterone could directly interact with the receptor thereby disturbing ligand binding. For example, progesterone was reported to interact with an extracellular site of the major brain nicotinic acetylcholine receptor, thus leading to inhibition of acetylcholine-evoked currents ($IC_{50} \approx 10$ μM) [10]. In the case of GPCRs which we have studied, several-fold higher progestin concentrations were needed to interfere with the ligand binding than to affect the ligand-induced calcium signaling. Our results are in contrast to those of Grazzini et al. who suggested a direct high-affinity interaction between 5β-pregnane-3,20-dione and the human oxytocin receptor [8]. These authors measured an inhibition of [^{3}H]oxytocin binding to the human oxytocin receptor in CHO membranes by 5β-pregnane-3,20-dione with $K_i = 32 \pm 5$ nM whereas we observed effects on ligand binding with 5β-pregnane-3,20-dione only at concentrations in the high micromolar range. (2) Progesterone may also influence receptor properties by changing the membrane fluidity. In fact, the membrane fluidity was slightly increased in membranes at 450 μM progesterone. This change in fluidity may contribute to the reduction of the ligand binding activity of the human oxytocin receptor in membranes of HEK-OTR or CHO-OTR cells. However, we observed no changes in the membrane fluidity at progesterone concentrations that affected the ligand-induced calcium signaling of GPCRs. (3) A well-known progesterone binding protein is the multidrug resistance P-glycoprotein [11] which acts as an energy-dependent drug efflux pump that reduces drug cytotoxicity when overexpressed (reviewed in [12]). Besides their role in detoxification, MDR P-glycoproteins are involved in intracellular sterol transport [13]. Progesterone inhibits the activity of P-glycoprotein and in turn blocks the cholesterol biosynthesis thus leading to the accumulation of cholesterol precursors such as lanosterol [2]. We show here that lanosterol itself did not affect the calcium signaling of the oxytocin receptor. Steroids like DHEA and pregnenolone are strong inhibitors of MDR P-glycoprotein mediated drug efflux [13] but they had little effect on calcium signaling of GPCRs in this study. Thus, an action of progesterone via this P-glycoprotein seems to be unlikely although an effect of progesterone on other related transporters cannot be excluded.

Our findings might be explained by the following observations. (1) Progesterone at concentrations used in our study has been reported to cause a rapid decline in the cholesterol level of caveolae, i.e. sphingomyelin/cholesterol-rich subdomains of the plasma membrane [14]. The signaling of the oxytocin receptor [9] and many other GPCRs (Burger et al., unpublished) depends on cholesterol. As many GPCRs and other molecules of the signaling cascade are enriched in caveolae [15], alterations in the cholesterol content of these microdomains by progesterone could lead to changes in the calcium signaling of GPCRs. (2) Progesterone could act on later steps of the signaling cascade. It may regulate the calcium channels of the plasma membrane or the membrane of the endoplasmic reticulum. This has been reported for intestinal smooth muscle cells where progesterone and 5β-pregnane-3,20-dione decreased voltage-gated calcium currents [16].

Conclusively, the progesterone doses which are required to affect the signaling function of receptors are higher than the progesterone levels found in plasma or in non-steroidogenic tissues such as the human myometrium [17]. Thus, our data do not support a non-genomic progesterone action within non-steroidogenic tissues. On the other hand, exceedingly high progesterone concentrations have been measured in specialized steroidogenic tissues, such as the placenta and corpus luteum. Near term, the human placenta secretes upward of 300 mg of progesterone daily. The progesterone content of this organ was shown to be 7 μg/g wet tissue [18]. In human corpus luteum, progesterone concentrations were cycle-dependent and reached peak levels of about 25 μg/g tissue

shortly after ovulation and in the early luteal phase [19]. Rough calculations show that all these values are within the range of the progesterone concentrations which were effective in our study. Thus, in steroidogenic cells as well as in their environment, progesterone might influence the effect of the ligands via non-genomic modulation of the ligand-induced calcium responses. In these tissues, the oxytocin system has also been discussed to play an important physiological role. For example, in the primate ovary, oxytocin is reported to function as a paracrine mediator in the luteinization process [20]. Moreover, during the course of human parturition, an enhanced expression of oxytocin receptors in chorio-decidual tissues was reported to occur [21]. Further studies are required to analyze the level of non-genomic action relative to the classical genomic effects of progesterone in reproductive tissues.

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